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Comparison of Akt/mTOR Signaling in Primary Breast Tumors and Matched Distant Metastases

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Abstract

Background—The Akt/mammalian target of the rapamycin (mTOR) signaling pathway represents a promising target for cancer therapy. The phosphorylation status of Akt and of mTOR's phosphorylation target eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) is often used to assess the activity of Akt and mTOR signaling. The purpose of this study was to determine whether primary tumors differ from their metastasis in their expression of pAkt and p4E-BP1.

Methods—Primary breast tumors and their distant metastases surgically resected from the same patients were evaluated with immunohistochemical analysis (IHC) for pAkt (Ser473) and p4E-BP1 (Ser65). The agreement between the IHC results for the primary tumor and metastases was evaluated with Cohen kappa (κ).

Results—Most primary breast tumors and metastatic tumors expressed pAkt (76% of each). Of the 23 matched evaluable pairs, however, 11 (47.8%) had discordant IHC results (κ −0.31; 95% confidence interval [CI], −0.49 to −0.13). Similarly, although most of the primary and metastatic tumors were positive for p4E-BP1 (75% and 74%), of the 23 matched evaluable pairs, 8 (47.8%) were discordant (κ 0.10; 95% CI, −0.33–0.52).

Conclusions—In this series, most primary breast tumors and metastases expressed pAkt and p4E-BP1 by IHC. Concordance between IHC findings in primary tumors and metastases was poor, however. Further work is needed to determine whether this reflects true biological heterogeneity or poor reproducibility of IHC with phosphospecific antibodies, and to identify which biomarkers can be assessed most reproducibly in primary tumors to predict activity of Akt/mTOR signaling and sensitivity to pathway inhibitors.

Keywords

mammalian target of rapamycin; biomarkers; metastases; Akt; targeted therapy

The Akt/mammalian target of rapamycin (mTOR) signaling pathway is a central regulator of cell growth and proliferation. Akt/mTOR signaling is aberrantly activated in several tumor types, including breast cancer, through mutations or decreases in expression of *PTEN* (phosphatase and tensin homolog deleted from chromosome 10), mutations in phosphatidylinositol 3-kinase (PI3K), or activation/amplification of growth factor signaling.

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[1,2] mTOR exists in at least 2 distinct functional complexes (TORC1 and TORC2).[3,4] Activation of mTORC1 results in phosphorylation of its effectors eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and S6K1, while mTORC2 is believed to regulate Akt Ser473 phosphorylation. Akt Ser473 phosphorylation is thought to precede Akt Thr308 phosphorylation by PDK1, and is important in fully activating Akt.[3] Both high levels of pAkt and of p4E-BP1 have been associated with a poor prognosis.[5,6] Thus, the Akt/mTOR signaling pathway is an especially promising target for breast cancer therapy.

Inhibitors of PI3K and Akt are now in early clinical development. The mTOR inhibitor rapamycin and its analogs have been tested actively in clinical trials for the past few years and have shown preliminary promise of efficacy in several tumor types.[7–9] Treatment with the rapalogue temsirolimus was associated recently with a survival advantage over interferon-alpha in a phase 3 clinical trial in advanced renal cell carcinoma (CCI-779, Wyeth, Madison, NJ).[10]

Most studies of mTOR inhibitors have shown clinical benefit for a small subgroup of patients. Notably, most of these studies were conducted in the metastatic setting, without selection of patients for activity of Akt/mTOR signaling. In preclinical work, tumors that have activation of the PI3K/Akt pathway have been sensitive to rapamycin; however, the value of pAkt as a predictive marker has not been validated in clinical trials.[11,12]

There remains a pressing need to identify markers that can determine the activity of Akt/mTOR signaling in tumors, and to determine whether marker assessment in the primary tumor accurately reflects the status of metastases arising from the same tumor. In this study we sought to determine whether primary tumors differ from their metastasis in expression of pAkt and p4E-BP1.

Materials and Methods

Patient and Tumor Characteristics

Patients who had breast cancer metastases surgically resected with palliative or curative intent were identified by searching databases maintained in the Departments of Surgical Oncology, Thoracic Surgery, Orthopedic Surgery, and Neurosurgery at the University of Texas M. D. Anderson Cancer Center. We then identified patients who had both their primary tumor and a distant metastasis surgically resected at the center. The study was limited to 29 patients for whom tissue blocks were available and viable tumor was identified on pathologic analysis. The study was approved by the Institutional Review Board.

The median patient age was 43 years (range, 26–76). At primary breast cancer diagnosis, 7 patients had stage I disease, 14 stage II, 6 stage III, and only 2 stage IV. Of the 28 patients who underwent axillary staging, 18 had lymph node-positive disease. Of the 23 patients for whom estrogen receptor (ER) status was known, 9 had ER-positive disease, and of the 21 for whom progesterone receptor (PR) status was known, 11 had PR-positive disease. Eighteen patients received adjuvant chemotherapy for their primary tumor; 10 received neoadjuvant chemotherapy. All patients who underwent adjuvant chemotherapy received an anthracycline-based regimen, and 4 also received an adjuvant taxane. Seven patients received adjuvant tamoxifen.

Median time to diagnosis of distant disease was 31 months (range, 0–153 months). Six patients underwent resection of their metastases with curative intent; all other patients underwent surgery with palliative intent. Distant metastases were excised from bone (12 patients), brain (11), lung (3), liver (2), or peritoneum (1). Ten patients received chemotherapy, 15 received

endocrine therapy, and 4 received radiation therapy to the site of metastasis before metastetectomy.

Immunohistochemical Analysis

Briefly, tissue samples were processed and embedded in paraffin. The paraffin-embedded samples were cut into 4-micron sections and placed on poly-l-lysine-coated slides. Sections were deparaffinized in xylene, rehydrated through graded alcohols, and transferred to phosphate-buffered saline solution (PBS). Immunohistochemical (IHC) detection of pAkt and p4E-BP1 was performed by using pAkt (Ser473; #9277) and p4E-BP1 (Ser65; #9451) rabbit polyclonal antibodies from Cell Signaling Technology (Danvers, Mass). Sections were placed in a preheated antigen retrieval solution (Dako, Carpinteria, Calif) in a steamer for 20 minutes. All samples were then blocked in 3% H_2O_2 in methanol for 15 minutes and rinsed with PBS. Slides were then placed in a Sequenza slide staining system (Thermo Fisher Scientific, Waltham, Mass) and blocked in 1% normal goat serum for 20 minutes. Slides were incubated overnight at 4°C with pAkt (1:100 concentration) or p4E-BP1 (1:50 concentration). This was followed by assay with a standard avidin-biotin peroxidase complex ABC Elite Kit (Vector Laboratories, Burlingame, Calif). Slides were developed with diamino benzidine (Zymed Laboratories, Carlsbad, Calif) and counterstained with 10% hematoxylin. IHC conditions were optimized using cell pellets from cell lines known to be positive or negative for pAkt (MDA-MB-468 and MDA-MB-435 cells) and p4E-BP1 (MCF7 cells cultured in the absence and presence of 100 nM rapamycin). After IHC conditions were established, the discrimination of samples positive and negative for pAkt and p4E-BP1 was confirmed by IHC of breast tumors previously shown to be positive or negative for pAkt and p4E-BP1.[5]

Quantitation and Statistical Analysis

For IHC quantitation, intensity (+, ++, and +++) and frequency were added to group the expression into 3 categories. Tumors with no staining or ≤10% of cells with (+) staining were scored as 0, tumors with >10% of cells with (+) staining or ≤20% of cells with (++ / +++) staining were scored as 1, and tumors with >20% of cells with (++ / +++) staining were scored as 2.[5] The IHC results were interpreted by 2 experienced breast pathologists (A.S., A.N.S.). pAkt and p4E-BP1 status was assigned according to a dichotomous scoring system: negative (score 0) or positive (score 1 or 2).

Discordance between IHC results for the primary tumor and metastasis or between the markers was represented as percentage discordance (number discordant/total number), with a 95% confidence interval (CI), including continuity correction. Agreement between sites or markers was evaluated with Cohen kappa (κ).

Results

To determine whether primary tumors differ from their metastasis in their Akt/mTOR signaling, we assessed the expression of pAkt and p4E-BP1 on matched surgical samples from 29 patients with breast cancer. Twenty-five primary tumor samples and 26 metastases were evaluable for pAkt immunostaining. Nineteen (76%) of 25 primary tumors and 20 (76%) of 26 metastatic tumors were positive for pAkt. Of the 23 matched evaluable pairs, however, 11 pairs were discordant (47.8%, 95% CI, 0.27–0.69) (Table 1). Examples of discordant samples are shown in Figure 1. The κ value was −0.31 (95% CI, −0.49 to −0.13), consistent with agreement less than expected by chance.

Of the 29 pairs of matched samples, 24 primary tumor samples and 27 metastases were evaluable for p4E-BP1 immunostaining. Eighteen (75%) of 24 primary tumors and 20 (74%) of 27 metastatic tumors were positive for p4E-BP1. Of the 23 matched evaluable pairs, 8 pairs

were discordant (34.8% discordance; 95% CI, 0.17–0.57). The κ value was 0.10 (95% CI −0.33–0.52), consistent with slight agreement.

Of the 24 primary tumors for which both pAkt and p4E-BP1 results were available, 5 tumors had discordance between the expression of these 2 markers (20.8% discordance; 95% CI, 0.08–0.43). Of the 26 metastases for which both pAkt and p4E-BP1 results were available, 7 were discordant (26.9% discordance; 95% CI: 0.12–0.48). Thus, the expression of the 2 markers showed moderate agreement in primary tumors (κ: 0.41; 95% CI, −0.01–0.84), and fair agreement in metastases (κ: 0.28, 95% CI, −0.13–0.69).

The interobserver variability for these markers was determined by comparing the independent scores by 2 pathologists. The interobserver variability for pAkt was 0.7939 (95% CI = 0.6019–0.9860). The interobserver variability for p4E-BP1 was 0.7195 (95% CI = 0.4893–0.9497). Thus, both markers showed substantial interobserver agreement.

Discussion

The Akt/mTOR signaling pathway is being actively pursued as a therapeutic target for cancer therapy. There is an urgent need, therefore, to identify robust markers that can determine the activity of Akt/mTOR signaling in tumors. pAkt has been widely studied as a marker of activity of the Akt signaling pathway, while mTOR's phosphorylation target p4E-BP1 is pursued by many as a marker of mTORC1 activity. It remains unknown, however, whether assessment of these markers in the primary tumor accurately reflects the status of metastases arising from the same tumor. We found that, although most of the primary and metastatic tumors in our study were positive for pAkt and p4E-BP1 by IHC, there was poor concordance between the primary tumors and metastases.

Activation of Akt/mTOR signaling in primary tumors is thought to represent a relatively aggressive phenotype. pAkt expression has been evaluated in a variety of primary tumor types.[5,13–22] Most of the published literature suggests that pAkt expression is a marker of poor prognosis in several tumor types, and is predictive of endocrine resistance in breast cancer.[13,14,19,20,22–24] The prognostic value of p4E-BP1, in comparison to that of pAkt, has been understudied. Castellvi et al.[6] recently studied the expression of pAkt and p4E-BP1 as well as EGFR, HER2, pS6K1, and pS6 in ovarian cancers and found that only p4E-BP1 demonstrated prognostic significance. Using reverse-phase proteomic arrays, Petricoin et al.[25] found that high pAkt and p4E-BP1 levels were associated with poor disease-free and overall survival in rhabdomyosarcoma. The findings of Zhou et al.,[5] who examined 165 invasive breast cancers by IHC, were similar to ours in that 74% of invasive tumors expressed pAkt and 72% expressed p4E-BP1. On univariate analysis, patients whose tumors expressed high levels of pAkt and p4E-BP1 tended to have shorter disease-free survival. Taken together, these data suggest that pAkt and p4E-BP1 play critical roles in cancer progression and indeed represent promising therapeutic targets.

Akt/mTOR signaling is being actively pursued as a target of cancer therapy. Inhibitors of PI3K and Akt are now entering into clinical trials. Rapamycin, which inhibits mTOR by binding FK binding protein 12, is already approved for use as an immunosuppressive agent in transplant patients and is now being investigated as an antitumor agent. Several rapamycin analogs, including temsirolimus, everolimus (RAD001; Novartis Pharma, Basel, Switzerland), and AP235673 (Ariad Pharmaceuticals, Cambridge, Mass), are well on their way in clinical trials. Encouragingly, treatment with temsirolimus was associated with a survival advantage over interferon-alpha in a phase 3 clinical trial in advanced renal cell carcinoma, demonstrating the potential clinical utility of mTOR inhibitors.[10] In most clinical trials performed in unselected cancer populations, however, mTOR inhibitors as single agents have shown only modest

efficacy. In the phase 2 trial of temsirolimus in breast cancer, for example, the objective response rate was 9.2%.[8] Thus, markers that can predict response to inhibitors of Akt/mTOR are needed to help select patients who are most likely to respond to these targeted therapies. pAkt status could potentially be used to select patients who have tumors more likely to be 'driven by' or 'addicted to' Akt activation, and thus are most likely to benefit from PI3K or Akt inhibitors. Akt activation has been associated with sensitivity to rapamycin and its analogs.[11,12,26] Whether these markers have predictive value in the clinic has not yet been demonstrated.

Metastatic tumors often are not easily accessible for biopsy, and thus markers that can be assessed reliably in the primary tumor are desirable. An ideal marker for clinical use would be stable in its expression over time, and its expression in the primary tumor would reflect its expression in metastatic sites. Indeed, gene profiling studies have shown that primary breast tumors are strikingly similar to distant metastases in the same patient.[27] HER2 expression has been widely studied, and is usually concordant between the primary tumor and different metastatic sites.[28–30] On the other hand, there are several reasons why there could be true biological differences in biomarker expression between primary tumors and metastases: 1) clonal divergence of biomarkers due to metastases arising from clonal expansion of primary tumor cells bearing additional genetic alterations; 2) differential modulation of gene expression and cell signaling in primary tumors versus metastases by the microenvironment; and 3) gain of additional aberrations in gene expression and cell signaling due to selection pressures imposed by adjuvant systemic therapy.[31] Our study suggests that, unlike HER2, pAkt and p4E-BP1 expression differs in primary tumors and their metastases. Interestingly, we expected that discordance, if any, would reflect higher Akt/mTOR signaling in metastases due to the increased metastatic potential of clones with activation of this pathway or due to selection pressure of systemic therapy selecting for increased Akt/mTOR signaling.[32] Instead, we found that the same number of patients had activation of Akt/mTOR signaling in their primary tumor and not in the metastasis as had activation in their metastasis but not in the primary. Further study is needed to determine whether our finding represents a true biological discordance and, if so, to identify its mechanism of development.

Another possibility is that the poor concordance between IHC findings in primary tumors and metastases in our study is a reflection of poor reproducibility of IHC with phosphospecific antibodies. There have been several concerns about the use of phosphoproteins as biological markers of tumor signaling. One major concern is that the staining and scoring of the findings of IHC with phosphospecific antibodies has not yet been standardized; no studies have yet assessed interlaboratory agreement for these markers. An even greater concern is the stability of phosphoproteins. Xenograft studies have shown a rapid decrease in pAkt at room temperature, with a calculated half-life of 20 minutes.[33] In a study conducted in paraffin-embedded samples from patients enrolled in a Southwest Oncology Group clinical trial, pAkt (Ser473) staining was observed only in clinical samples obtained as biopsies (9 of 13), not in those obtained as surgical samples (0 of 15).[33] Many other markers show tumor heterogeneity, however, and it is thought that there may be a gradient of mTOR activity due to central hypoxia in larger tumors, raising a concern about assessing markers of Akt/mTOR signaling on limited tumor samples obtained though core biopsy. In this study we elected to look at surgical samples from primary and metastatic tumors collected in the same institution to try to minimize variation in specimen processing as well as variability due to tumor heterogeneity. We acknowledge that there still will be inherent variation in tumor hypoxia times in this retrospective study because of differing surgical procedures (eg, breast conservation vs mastectomy vs liver resection with vascular occlusion), as well as variation in processing and fixation of tumor at different sites. Markers that are robust enough to remain reproducible in spite of these technical variations will be most useful in the clinical setting.

The main limitation of this study is its small sample size. This is inherent in our study group choice, as distant metastases from breast primaries are rarely surgically resected. It also should be noted that most of the primary tumors in our study were removed before systemic therapy, while most patients received systemic therapy after the diagnosis of metastasis. Although this study design is not optimal to capture de novo biological variations between primary tumors and metastases, comparison of biomarker expression in primary tumors and metastases already treated with systemic therapy better reflects the scenario in which patients with metastatic disease will undergo biomarker testing to assess eligibility for novel targeted therapies. Further study in larger cohorts is needed to determine whether there is actual discordance between Akt/mTOR signaling in primary and metastatic tumors, and whether this is a true biological phenomenon or reflective of technical variations in IHC staining of tumors retrieved from different sites. Further work is needed to compare tissue in paraffin with fresh-frozen samples, and to compare IHC with newer, more quantitative technologies such as reverse-phase proteomic arrays. Identification of robust biomarkers that can be utilized widely with low interlaboratory variability and can accurately assess aberrations in cell signaling and predict response to targeted therapies is critical to successful delivery of individualized cancer therapy.

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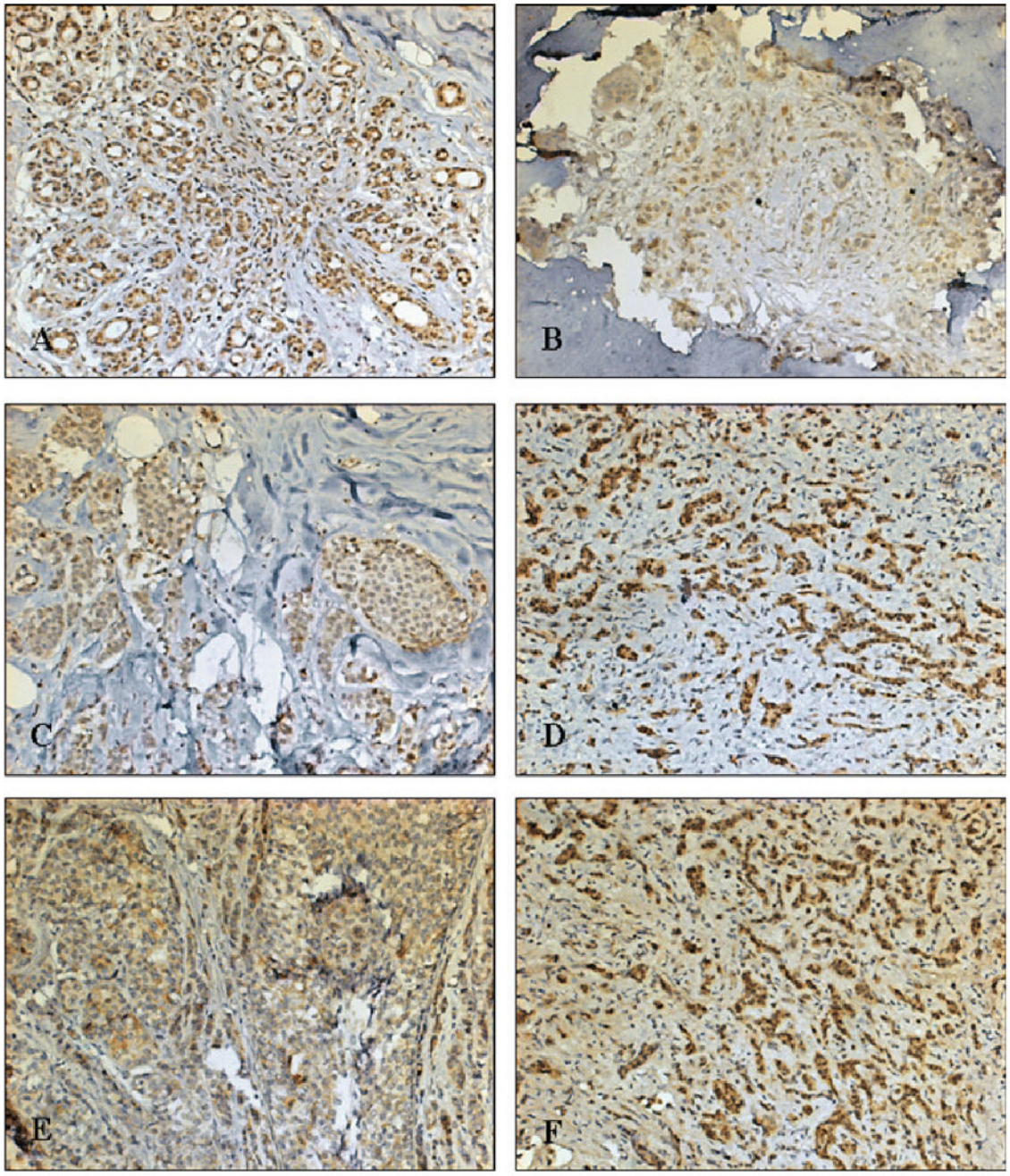

FIGURE 1.

Immunohistochemical staining (A–D) p4E-BP1 expression. (A) Primary breast tumor shows strong cytoplasmic and occasionally nuclear staining. (B) Bone metastasis of the same patient shows weak cytoplasmic staining. (C) Weak staining of the breast tumor and (D) strong staining of corresponding liver metastasis. (E,F) pAkt expression. (E) Weak cytoplasmic staining of the primary tumor. (F) Strong cytoplasmic staining of the corresponding liver metastasis. Note that slides C–F belong to the same patient. Original magnification, ×100.

TABLE 1

Comparison of the pAkt and p4E-BP1 Status in Primary Versus Metastatic Tumors

				Discordance		
		Metastasis negative	Metastasis positive	No. discordant/total no.	% Discordant	Kappa (95% CI)
pAkt	Primary negative	0	6	11/23	47.8	−0.31 (−0.49–0.13)
	Primary positive	5	12	—	—	—
p4E-BP1	Primary negative	2	4	8/23	34.7	0.10 (−0.33–0.52)
	Primary positive	4	13	—	—	—